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Exhibit 10 [Fifth Supplemental IDS -- February 6, 2006]

# EXHIBIT 10

Enz-7(P)(C3)



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TRANSLATIONS

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I, Jinmei Yin, hereby certify that the following is, to the best of my knowledge  
and belief, a true and accurate translation of a document entitled "Nucleic Acid  
Research Methods (part 2 of 2)" from Japanese into English.

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Nucleic Acid Research Methods (part 2 of 2)  
METHOD  
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## Membrane Filter Technique for DNA-DNA Hybridization

[see source for drawing]

Yasuo Imae\* and Ritsu Kuroda\*\*

### INTRODUCTION

Since it has become possible to separate and recombine two chains of DNA *in vitro*, it has become possible to investigate using DNA levels genetic homology. In addition, using the idiosyncrasies of DNA-DNA hybrid formation, it has become easy to perform analysis for specific DNA within many kinds of DNA mixtures.

Many methods for DNA-DNA hybridization have been developed, with the principle ones as McCarthy et al. DNA-agar technique<sup>1-3)</sup> and Denhardt et al. Membrane Filter Technique<sup>5-7)</sup>. Because the former requires a large amount of material, its use is not found in research which uses bacterial or phage DNA. On the other hand, the latter, for which small amounts of material are acceptable, excels at handling large amounts of material simple at one time, and because it is difficult to handle large amounts of DNA, the analysis of large size genome that are present within animal cell systems is correspondingly difficult. Here, in this paper, considering the principal limitations for phage and bacterial systems, a membrane filter technique is used which was performed in the laboratory by the writers.

Moreover, we will reference McCarthy and Church<sup>8)</sup> and Midgley<sup>9a)</sup> and De Ley<sup>9b)</sup> for a complete understanding for the annealing of nucleic acid.

### I. Fundamental Properties of DNA Renaturation

Because the reassociation of DNA chains is a reaction of two polynucleotide chains which are negatively charged and the higher the cation concentration the faster the reaction is promoted. In this instance, with a high salt concentration, because the  $T_m$  (DNA melting temperature) is raised, it is generally necessary to elevate the reaction temperature. Furthermore, the reaction is significantly dependent on the length of the DNA chain or the size of the genome. The theoretical foundations of the renaturation kinetics within solutions of DNA which have not been repeated and the effect of various factors can be found

in the detailed reports of Wetmer and Davidson<sup>10)</sup>, but, first, initially, considering the main points, there is introduced the fundamental properties of DNA-DNA hybrid formation. Even when using membrane filtering, by eliminating one or two points, because there is consideration given, fundamentally, to the same reaction formation method, let's reference the establishment of conditions for various experiments.

#### 1. Reaction Manner

The DNA renaturation reaction advances as a secondary reaction. That is, the mutually complementary DNA chains collide, and the stage at which the initial effective base pairs form is the rate controlling stage. The "zippering" reaction towards completion of the two-chain DNA which is occurring, advances at an usually rapid pace.

Let  $P$  be the concentration of a one chain DNA in the solution, if the renaturation reaction follows a secondary reaction, the following equation is established.

$$-d(P)/dt = k(P)^2/2 \quad (1)$$

( $t$ : reaction time,  $k$ : rate constant of the renaturation reaction)

By integrating, letting  $P_0$  be the initial concentration of the one chain DNA, and letting  $P$  be the concentration at time  $t$ ,

$$P_0/P = kP_0/2 t + 1 \quad (2)$$

Experimentally,  $P_0/P$  is linear with respect to the reaction time, and the rate constant  $k$  expresses that there are no changes according to differences in DNA initial concentrations<sup>10)</sup>, and it is confirmed that DNA renaturation follows a secondary reaction.

#### 2. Temperature Effects

The rate constant of the renaturation reaction expresses the temperature dependencies as shown in Figure 1, and generally, become maximum in the vicinity of ( $T_m - 25^\circ\text{C}$ ).

#### 3. Effects of DNA Fragment Size

Let  $L$  be the average nucleotide number of a one chain DNA molecule, and experimentally,  $k$  is proportional to  $L^{1/2}$ . Consequently, when there is a reaction in solution, the longer the DNA chain, the greater the reaction rate.

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Membrane Filter Technique for DNA-DNA hybridization

\* Figures 1, 5, 6, and 7 are based on the important points of charts in Wetmer and Davidson's article. Please be warned that these were plotted ignoring differences in the details of the conditions.

## Membrane Filter Technique for DNA-DNA Hybridization

[see source for figure]

[x axis] Reaction Temperature (°C)

Figure 1. Renaturation temperature dependency  
The reaction is performed with 1.0 M Na<sup>+</sup>

$$T_{mT4DNA} = 92^{\circ}\text{C}$$

$$T_{mE.coliDNA} = 98^{\circ}\text{C}$$

On the other hand, using the later described membrane filtering technique, conversely, in order to minimize the self-annealing within the solution of the added marked DNA, processing is performed to make L small.

A long chain of 12 nucleotides or more is necessary to impart the capability for DNA hybrid formation, and furthermore, in order to express some specificity, for the case of T4 DNA, 17 or more nucleotide chains are necessary<sup>11)</sup>. In addition, when the temperature is low, the specificity is weak (Figure 2), and when the chain length is short, and the efficiency of the annealing reaction at temperatures that are very much below  $T_m$  become worse (Figure 3). Consequently, by holding accurately the specificity, and making the reaction rate high, even the DNA chain becomes small, and there must be a length of 200 or more nucleotides.

[see source for figure]

[y axis] % for preparing hybrid  
[x axis] Reaction Temperature (°C)

Figure 2. Specificity<sup>11)</sup> with renaturation temperature

[see source for figure]

[y axis] Correlation % for preparing hybrid  
[x axis] Reaction Temperature (°C)

Figure 3. Reaction properties with size of DNA fragment<sup>11)</sup>

With respect to 100  $\mu\text{g}$  of mouse DNA, added <sup>14</sup>C mouse DNA (0.1  $\mu\text{g}$ ) which changed the size and then reacted. n: degree of polymerization

#### 4. Effect of genome size and GC weight

Using the same DNA concentration, the renaturation rate for phage DNA was higher than for bacterial DNA<sup>12)</sup>. Let N be the number of the base pairs per genome, and it is established that k is proportional to 1/N. Figure 4 is a plot with respect to genome size of the value of the reaction rate constant for various types of DNA, and the above-mentioned relationships is understood to be established. Consequently, for any lengthening of the reaction time or increase in the DNA concentration, it is necessary to take countermeasures.

[see source for figure]

[x axis] Genome Size (daltons)

Figure 4. Genome size and reaction rate constant<sup>10)</sup>  
All the DNA that was used, had a size of approximately 7S

In addition, for the same level of genome size, the larger the amount of DNA for a given GC weight, the faster the reaction rate, and when the GC percentages are 34, 41, 50, and 64, the complementary value of  $k$  is 0.69, 0.81, 0.98, 1.27<sup>10</sup>. Consequently, as the renaturation reaction evolves, it is understood that initially there occurs a reaction from DNA fragments which have high GC content.

#### 5. Effect of pH, ion strength and viscosity

The reaction rate, for renaturation, becomes higher, when in the vicinity of pH 7 (Figure 5) and when the ion strength is high (Figure 6). In addition, the value of  $k$  becomes small according to the rise in the viscosity of the reaction liquid.

## II. DNA-DNA hybridization

The DNA-DNA hybridization method that uses a membrane filter is an improved version of that developed as a DNA-RNA hybridization method by Gillespie and Spiegelman<sup>13</sup> that can be applied to DNA as well. Single-stranded DNA differs from RNA and double-stranded DNA in that it is well-adsorbed by the membrane filter. Consequently, it is necessary to prevent the single-stranded DNA from being non-specifically adsorbed into the membrane filter during the hybridization reaction. Denhardt<sup>5</sup> performed the hybridization reaction after first covering a membrane filter to which single-stranded DNA has been affixed with albumin and a synthetic polymer and eliminated the non-specific adsorption of single-stranded DNA in solution. Legault-Démare et al.<sup>6</sup> performed hybridization in dimethylsulfoxide and prevented non-specific adsorption. In addition, Warneer and Cohen<sup>7</sup> wash the filter with a low ion strength, high pH buffer solution after hybridization to wash away the non-specifically adsorbed DNA and to reduce the background. These three methods have already been presented in detail<sup>14</sup>, so please refer to the literature. Herein is presented a method that further improves upon the Denhardt method employed by the authors, et al.

[see source for figure]

Figure 5. pH dependency of the renaturation reaction<sup>10</sup>  
React the T4 DNA (20S) with 0.4M Na<sup>+</sup>. Temperature was approximately (T<sub>m</sub>-25°)

[see source for figure]

[x axis] [Na<sup>+</sup>] concentration (M)

Figure 6. Effect of the ion strength on the renaturation reaction<sup>10</sup>  
React under the same conditions as with Figure 5 (pH 7.0)

### 1. DNA preparation

#### A. DNA affixed to the membrane filter

The bacteria DNA is extracted by the Marmur method<sup>15</sup>, the Thomas method<sup>15</sup>, or a combination of both. The RNA and protein are eliminated insofar as possible. Phage DNA is adjusted through phenol extraction<sup>17</sup>.

#### B. The DNA to be introduced to the reaction liquid

Radioactive DNA is used and traced by its radioactivity, so there is no need for the DNA to be pure. It is a problem if the RNA produces competitive inhibition to the point where it infiltrates. The proteins, lipids, etc. do not affect the reaction. Accordingly, after fractionating the DNA extracted from the bacteria with alkalis and surfactants with sucrose concentration gradient centrifugation, it is possible to use it immediately as well simply by eliminating the sucrose by means of dialysis. The sample DNA is made into single-strand DNA by means of heat processing or such immediately before use.

[see source for figure]

[x axis] Simple sugar concentration (%)

Figure 7. Effect of viscosity on renaturation reaction<sup>19</sup>  
Conditions same as for Figure 6.

## Membrane Filter Technique for DNA-DNA Hybridization

**2. Affixing the DNA to the membrane filter**

To be prepared:  $10 \times \text{SSC}$  ( $10 \times$  concentration of SSC; the SSC is  $0.15_M \text{ NaCl}$ ,  $0.015_M$  sodium citrate). Membrane filter: 20–25 mm diameter, Millipore HA 0.45  $\mu$ , Schleicher-Schuell Type B-6 coarse or Sartorius membrane filter MF 50. Solution scintillation counter class vial (hereinafter referred to as "vial").

Method: Double-stranded DNA is heat processed for 5 minutes at  $100^\circ\text{C}$  in  $0.1 \times \text{SSC}$  and then quenched and made into single-stranded DNA. At this time the DNA concentration is made to be as low as possible, and renaturation is kept low. A certain amount is taken and made to a concentration of  $6 \times \text{SSC}$ , and the volume is made to be 3–5 ml. This is then slowly filtered using a membrane filter that has been soaked in the  $6 \times \text{SSC}$  in advance. A flow rate of approximately 3 ml per 30 seconds is acceptable, but when the DNA molar weight is less than  $6 \times 10^6$  Daltons then the filtration must be performed extremely slowly (3 ml per 10 minutes or more) or else the efficiency of adsorption to the filter will decrease. After washing the filter at the same rate as described above with 5 ml of  $6 \times \text{SSC}$ , this is transferred to a vial and dried overnight in a desiccator with silica gel under reduced pressure. The filter is processed for 2 hours and  $80^\circ\text{C}$  under reduced pressure in a vacuum dryer as it is in the vial, and the DNA is impressed onto the filter. When a vacuum dryer is not available, it is acceptable to heat process for 2–3 hours at  $80^\circ\text{C}$  in an ordinary dryer, then immediately transfer it to a desiccator, and then cool it with silica gel. The filter upon which the DNA is affixed can be used over a long period of time as long as it is stored in a dry state.

**3. Pre-incubation**

To be prepared: PM; 0.02% ficoll (Pharmacia, average molecular weight 400,000), 0.02% polyvinylpyrrolidone (PVP) (Sigma, average molecular weight 360,000), 0.02% bovine serum albumin (BSA) (Sigma, Fraction V), 0.05% sodium dodecyl sulfate (SDS) dissolved in  $3 \times \text{SSC}$ .

Method: 1 ml of PM is added to a vial containing the DNA filter, and this is completely covered, immersed in  $65^\circ\text{C}$  water, and kept for six hours. The authors et al. kept it immersed pressed under a metal basket they had made. It is preferable to perform DNA-DNA hybridization immediately after holding it at this temperature, but if necessary it can be performed after refrigerating for one night ( $4^\circ\text{C}$ ) without reducing efficiency that much.

**4. DNA-DNA hybridization**

To be prepared: a vial.  $10 \times \text{PM}$ ; including ficoll concentrated to  $10 \times \text{PM}$  in  $3 \times \text{SSC}$ , PVP, BSA, and SDS.  $3 \times 10^{-3}_M$  Tris-hydrochloric buffer solution (pH 9.4).

Method: While pre-incubating, 70  $\mu\text{l}$   $10 \times \text{PM}$  and 630 ml of single-strand DNA (in  $3 \times \text{SSC}$ ) from the marked sample is placed into a separate vial in advance. The pre-incubated filter is taken out, and after dabbing the mouth of the vial with plenty of PM, this is transferred to the vial containing the sample, and this is incubated for 12 hours at  $65^\circ\text{C}$  according to the method described previously. After incubation, the filter is rinsed 3 times with the  $3 \times 10^{-3}_M$  Tris-hydrochloric buffer solution (pH 9.4), and each side of the filter is washed with 50 ml of the same solution while further aspirating. After drying under an infrared lamp, it is counted using a toluene scintillator. As for total count volume of the marked DNA added, a certain amount is placed directly on the filter in a low salt concentration state and it is measure in a similar manner after drying.

**5. Supplement**

A. Fundamentally, the DNA-DNA hybridization method using a membrane filter follows the insoluble renaturation kinetics described in I., and we will not restate detailed parameters here. However, as seen in Figure 8, significant dependency upon the volume of the reaction solution is evidenced<sup>7)</sup>, and so the amount reaction liquid should be reduced as much as possible and it is more efficient if the volume is kept to just the amount required to completely immerse the filter.

B. The hybrid formative ability is determined according to the genome size and the degree of self-annealing. If the genome size is large then it is better to increase the quantity of the DNA to be affixed to the filter. Figure 9 shows the relationship between the change in efficiency and genome size when the quantity of DNA changes. Even if it is said that it is better to have a larger quantity of DNA affixed, if the amount reaches 100  $\mu\text{g}$  DNA per filter or more, then the speed of final washing with the Tris-hydrochloric buffer solution is extremely reduced and too much time is required, so this is not practical. When the quantity of DNA must be increased in order to increase efficiency, another method is to add several DNA filters to a single vial. However, in this case it is necessary to increase the quantity of the reaction liquid so that all of the filters are completely immersed.

[y axis] Percent creating hybrid  
[x axis] Quantity of the reaction liquid (ml)

Figure 8. The effect of the quantity of the reaction liquid in the membrane filter method<sup>7)</sup>

T4 DNA has been affixed to the filter at the quantities shown in the figure.  $^{32}\text{P}$ -T4 DNA (sonically processed) was reacted in SSC for 26 hours at  $60^\circ\text{C}$ .

[y axis] Percent creating hybrid  
 [x axis] T4 DNA ( $\mu\text{g}/\text{filter}$ ) / *E. coli* DNA ( $\mu\text{g}/\text{filter}$ )  
 Quantity of the DNA affixed

Figure 9 The relationship between the quantity of the DNA affixed to the filter and reaction efficiency

Please refer to the body text for the reaction conditions. a)  $^3\text{H}$ -T4 DNA; 0.1  $\mu$ , 952 cpm (size is 7-10S). b)  $^3\text{H}$ -*E. coli*; 0.05  $\mu$ , 1,600 cpm (processed in 0.5N NaOH for 7 minutes at 100°C.).

Table 1 shows the usual fixation quantity of DNA used in the laboratory of the authors et al. and the efficiency of hybridization.

C. In order to prevent the self-annealing of the added marked DNA in solution, it is acceptable for either the quantity of the marked DNA to be much lower than the quantity of the affixed DNA, or for the molecular weight of the marked DNA to be reduced by some means. Alkali processing is simple and convenient for reducing the molecular weight. If the DNA has a molecular weight of  $10^7$ - $10^8$  Daltons, then heating for 7 minutes at 100°C in 0.5N NaOH will result in a size of approximately  $10^5$ - $10^6$  Daltons, and the efficiency of hybridization will be the best. Table 2 shows the time of alkali processing and the change in the size of the DNA <sup>16)</sup>. After alkali processing, this is neutralized and added to the reaction liquid.

Sonication is effective for lowering the molecular weight of the DNA. Table 3 shows the change in efficiency with sonic processing.

D. By adding the SDS to the reaction liquid, there is no effect on the efficiency of hybridization, but the membrane filter becomes pliable and easy to handle. In addition, the "wetness" of the glass wall of the vial improves, and it becomes possible to eliminate the change in the volume of the reaction liquid that generates condensation of moisture on the glass wall during the reaction, so reproducibility improves remarkably.

Table 1. Genome size and efficiency of hybridization

[see source for table]

Affixed DNA / Genome size (Daltons) /  
 mg/filter / % hybrid <sup>1)</sup>

a) The label DNA is fractionated to  $10^5$ - $10^6$  Daltons and measured at a quantity of 0.1  $\mu\text{g}$  or less



## Membrane Filter Technique for DNA-DNA Hybridization

Even if a relatively low count is used, the deviation is  $\pm 5\%$  or less when duplicated, and in experiments where many points will be taken, duplication is not particularly necessary. It is acceptable to have the background (non-specific adsorption of to a filter to which no DNA is affixed) with this method at 0.1% or less. When SDS is added the background decreases to 0.5%–0.05% with the method of Warnaar and Cohen <sup>19)</sup>, but with the method described above, it is 0.1% or lower regardless of the presence of SDS. The disadvantage of having SDS added is that there is a bit more separation of the DNA from the filter, but in most experiments it is of a degree that can be ignored (it is said that when SDS is added to 0.1%, approximately 5% of the DNA affixed at 60°C and 20 hours separates) <sup>19)</sup>.

E. When handling DNA where the genome size is extremely small, the quantity of the affixed DNA and that of the marked DNA must be significant or self-annealing will occur quickly and the efficiency of hybridization will be reduced.

F. Experimentally, even if Tris-hydrochloric buffer solution (pH 9.4) is not produced, if Trizma base is dissolved as is in distilled water then the pH will approach 9.4 and it will be acceptable to use it as is.

Table 2. Alkali processing time and average DNA size <sup>18)</sup>

Processing time <sup>a)</sup> / Average DNA size (nucleotide) <sup>b)</sup>  
[see source for table]

0 minutes  
3 minutes  
7 minutes  
15 minutes

a) 0.5N NaOH processed at 100°C

b) Fractionated with alkaline sucrose concentration gradient centrifugation with the peak position of the acid-insoluble nucleotide adjusted for the number of nucleotides

Table 3. Change in the efficiency of hybridization due to sonic processing

Processing condition <sup>a)</sup> / % hybrid <sup>b)</sup>  
[see source for table]

No denaturation  
Alkaline denaturation  
Sonic processing / alkaline denaturation  
Sonic processing / thermal denaturation

a) Sonic processing was performed for 30 seconds at the highest output with the MES Ultrasonic Disintegrator

b) The marked  $\lambda$ DNA (0.06  $\mu$ g, 850 cpm) was hybridized with the 1.5 mg/ filter  $\lambda$ DNA

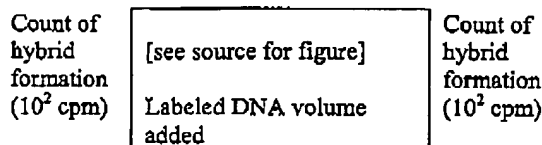


Figure 10. Quantitative characteristic of hybridization. Refer to the text for the condition of reaction. a) T4 DNA 2  $\mu$ g is fixed.  $^{14}$ C-T4 DNA: 750 cpm/0.16  $\mu$ g. b) *E. coli* DNA is fixed at 20  $\mu$ g.  $^3$ H-*E. coli* DNA: 1,000 cpm/0.025  $\mu$ g. ALL DNA are fragmented through alkali extraction.

### III. Quantitative characteristics and idiosyncrasy of DNA-DNA hybridization: Experiment using T4 and *E. coli* DNA

#### 1. Quantitative characteristics

When the DNA volume to be fixed is kept at a regular volume and the volume of labeled DNA to be added is changed, the result is shown is a straight line for a considerable range (Figure 10). That is, if a sufficient volume of DNA is fixed, the added labeled DNA will form hybrid at a certain rate. In case of T4 DNA, when 2  $\mu$ g DNA is fixed, the straight line is still maintained even if roughly 1  $\mu$ g DNA is added.

#### 2. Idiosyncrasy

Figure 11 shows that marked *E. coli* DNA hardly form hybrid with fixed T4 DNA. That is, the idiosyncrasy of DNA-DNA hybridization is very high.

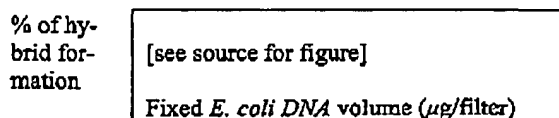


Figure 11. Homology between *E. coli* DNA and T4 DNA

$^3$ H-*E. coli* DNA (2,000 cpm, 0.05  $\mu$ g) and  $^{14}$ C-T4 DNA (2,000 cpm, 0.36  $\mu$ g) were added to the filter with fixed *E. coli* DNA in the same vial to see the reaction.

Figure 12 shows the result of competitive DNA. When the T4 DNA is fixed to a filter and the labeled DNA is competed against unlabelled DNA upon hybridization, the rate of labeled DNA hybridization will significantly reduced. The rate is the same when competed against *E. coli* DNA. Naturally, the DNA volume to be competed against shall be calculated based on the fixed DAN volume rather than the labeled DNA volume.

### IV. Other applications

#### 1. Identification of the complementary strands separated from T4 DNA

The idiosyncrasy shown in III-2 is recognized other than between different types of DNA. While the two complementary strands of T4 DNA can be separated through the CsCl density-gradient centrifugation using the difference in binding capabilities, the separated DNA strands will not form a hybrid with the same DNA strand portion but form hybrid only with the opposite DNA strand (Figure 13)<sup>21</sup>.

#### 2. Quantity of bacterial DNA within $\lambda$ dg DNA

It is assumed through genetic method etc. that about 50% if  $\lambda$ dg<sub>A-J</sub> DNA is  $\lambda$ DNA, and the remaining 50% is built into with *E. coli* DNA near galactose operon. When the labeled  $\lambda$ dg<sub>A-J</sub> DNA is hybridized with the filter fixed only with  $\lambda$ DNA and the filter fixed with both  $\lambda$ DNA and  $\lambda$ dg<sub>A-J</sub> DNA, unless there is a homology between *E. coli* DNA and  $\lambda$ DNA, the difference between the two filters shall correspond to the size of *E. coli* DNA. The diagram 14 shows the result<sup>22</sup> and the gap is about 30%, which is the same value as the value identified through genetic method etc. The resulting value is slightly smaller because there is a partial homology between *E. coli* DNA and  $\lambda$ DNA.<sup>23</sup>

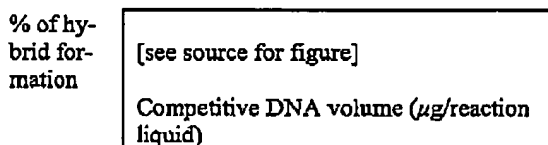


Figure 12. The effect of competitive DNA

T4 DNA 3  $\mu$ g was fixed and added with  $^3$ H-T4 DNA 0.1  $\mu$ g and unlabeled T4 DNA or *E. coli* DNA for the amount shown in the diagram for reaction.

## Membrane Filter Technique for DNA-DNA Hybridization

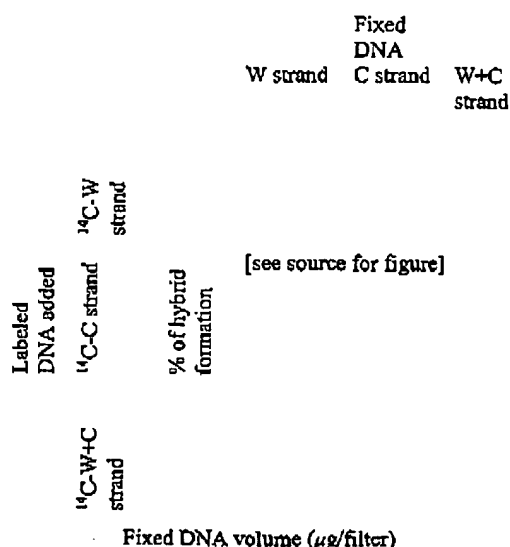


Figure 13. Hybridization in T4 DNA with separate strands<sup>21)</sup>

Using poly-UG, T4 DNA strand is separated into C strand and W strand and each was fixed onto separate filters.  $^{14}\text{C}$ -T4 DNA is also separated into C strand and W strand and each was added with 0.18  $\mu\text{g}$  (750 cpm) for hybridization. For W+C strand, both strands were added simultaneously.

### 3. Separation of specific DNA strand using DNA-DNA hybridization

Labeled DNA strand can be separated into complementary strands by first fixing DNA which has been separated into complementary strands and by separating the labeled DNA which is to form a hybrid with it.<sup>24)</sup> When T4 phage infected bacterium is labeled for a short period with  $^3\text{H}$ -thymidine and the DNA is processed with alkaline sucrose density-gradient centrifugation method, the resulting pattern can be seen as in diagram 15-a.

Count of  
hybrid  
formation  
( $10^2$  cpm)

[see source for figure]

$2\text{H-}\lambda\text{dg}_{\lambda\text{I}}$  DNA volume ( $\mu\text{g}/\text{reaction}$   
liquid)

Figure 14 Fixed quantity<sup>22)</sup> of *E. coli* DNA built into  $\lambda\text{dg}_{\lambda\text{I}}$  DNA

a) ---: Fixed with  $\lambda\text{DNA}$  10  $\mu\text{g}$ . -o-: Fixed with  $\lambda\text{DNA}$  5  $\mu\text{g}$  and  $\lambda\text{dg}_{\lambda\text{I}}$  DNA 5  $\mu\text{g}$ . After the reaction,  $3\text{H-}\lambda\text{dg}_{\lambda\text{I}}$  DNA (1,774 cpm/0.1  $\mu\text{g}$ ) and the competitive 20  $\mu\text{g}$   $\lambda\text{DNA}$  are added.

b) The count of hybrid on  $\lambda\text{DNA}$  is subtracted from the count of hybrid on  $\lambda+\lambda\text{dg}$  DNA filter.

Radioactivity  
( $10^2$  cpm/ml)

[see source for figure]

Cubic volume from centrifugation surface

Figure 15. Collection from the labeled DNA filter with hybrid formation<sup>24)</sup>

a) T4 infected bacterium was pulse-labeled with  $^3\text{H}$ -thymidine and analyzed with alkaline sucrose density-gradient centrifugation method. The parts shown in parenthesis are removed (-10S) and after hybridized with T4 DNA W strand and C strand, the filter was washed through regular method.

b) After the hybridization with W strand, solved out with 0.1 N NaOH-0.01M EDTA, and analyzed in the same method as a).

c) After the hybridization with C strand, analyzed in the same method as b).

Then 8 ml reaction liquid was made after the peak fraction is collected and processed with dialysis to conduct DNA-DNA hybridization. Next, W strand and C strand of T4 DNA are each fixed in the volume of 2  $\mu$ g onto thirty filters. All filters are added to the reaction liquid (it is recommended to cut an end of filters to distinguish W strand and C strand), washed through regular method after the hybridization, and the labeled DNA was solved out after soaking 10 minutes in 5 ml 0.1 N NaOH-0.01M EDTA. Figure 15-b and c show the result of DNA solved out from W filter and C filter after processed with alkaline sucrose density-gradient centrifugation method. As long as the size of DNA is about 10S, further fragmentation was not recognized during the operation. The collection rate was about 20% in both W strand and C strand.

Above method is very useful for handling samples in which the principle of "strand separation" can not be easily applied, as in the case of DNA during replication, or when the characteristics of specific DNA within a mixture is sought.

#### ENDNOTE

Recently Cot analysis, which utilized the fact that the DNA-DNA hybridization reaction occurs according to secondary formula and in which the DNA homology (and especially the repetition within one genome) is measured through the reaction rate, came to be used widely in the area of embryology etc. When the initial density of DNA is  $C_0$ , the reaction can be shown in the following formula based on (2) formula:

$$\frac{C}{C_0} = \frac{1}{1 + k C_0 t}$$

Accordingly, the ratio of the single strand in the reaction liquid ( $C/C_0$ ) is exactly a half when the multiplication of the initial density and the reaction time ( $C_0 t$ ) is  $1/k$ . As noted earlier, while the genome size  $k$  may fluctuate, the same variation can be seen when the  $C_0 t$  is used as a unit instead of  $k$ . Thus, we designate the method to find out the genome size or repetition volume within the genome through the measurement of  $C/C_0$  and  $C_0 t$  as Cot Analysis. For detailed discussion, please refer to the general description<sup>23)</sup> and the page written by Fujinaga (page 74) in this document.

#### LITERATURE

- 1) E.T. Bolton, B.J. McCarthy: *Proc. Acad. Sci.*, 48, 1390 (1962)
- 2) B.J. McCarthy, E.T. Bolton: *Proc. Acad. Sci.*, 50, 156 (1963)
- 3) D.B. Cowie, B.J. McCarthy: *Proc. Acad. Sci.*, 50, 537 (1963)
- 4) S. Falkow, R.V. Citarella: *J. Mol. Biol.*, 12, 1387 (1965)
- 5) D.T. Denhardt: *Biochem. Biophys. Res. Comm.*, 23, 641 (1966)
- 6) J. Legault-Demare, B. Desseaux, T. Heyman, S. Seror, G.P. Ress: *Biochem. Biophys. Res. Comm.*, 28, 550 (1967)
- 7) S.O. Warnaar, J.A. Cohen: *Biochem. Biophys. Res. Comm.*, 24, 554 (1966)
- 8) B.J. McCarthy, R.B. Church: *Ann. Rev. Biochem.*, 39, 131 (1970)
- 9a) J.B.M. Midgley: *Methods in Microbiology* (ed. J. R. Norris, D.W. Ribbons), 5A, 331, Academic Press (1971)
- 9b) J. DeLey: *ibid*, 5A, 311 (1971)
- 10) J.G. Wetner, N. Davidson: *J. Mol. Biol.*, 31, 349 (1968)
- 11) B.L. McConaughy, B.J. McCarthy: *Biochem. Biophys. Acta*, 149, 180 (1967)
- 12) J. Marmur, P. Doty: *J. Mol. Biol.*, 3, 585 (1961)
- 13) D. Gillespie, S. Spiegelman: *J. Mol. Biol.*, 12, 829 (1965)
- 14) Ichiro Odakagi: *Method in Nucleic Acid Research*, 13, 1085 (1968)
- 15) J. Marmur: *J. Mol. Biol.*, 3, 208 (1961)
- 16) C.A. Thomas, Jr., K.I. Berns, T.J. Kelly, Jr.: *Procedures in Nucleic Acid Research* (ed. G.L. Cantoni, D.R. Davis), p. 535, Harper & Row Publishers, New York (1966)
- 17) C.J. Thomas Jr., J. Abelson: *Procedures in Nucleic Acid Research, ibid*, p. 553
- 18) Tadao Kanemori (unpublished)
- 19) Yuji Tomiyama (Private communication)
- 20) Koji Okamoto (Private communication)
- 21) K. Sugimoto, T. Okazaki, Y. Imae, R. Okazaki: *Proc. Natl. Acad. Sci.* 63, 1343 (1969)
- 22) Y. Imae, T. Fukusawa: *J. Mol. Biol.*, 54, 585 (1970)
- 23) H. Yamagishi, A. Skalka: *J. Mol. Biol.*, 58, 417 (1971)
- 24) T. Okazaki, R. Okazaki: *Proc. Natl. Acad. Sci.*, 64, 1242 (1969)
- 25) Masami Muramatsu: *Method in Nucleic Acid Research*, 17, 609 (1972)

## Membrane Filter Technique for DNA-DNA Hybridization

## Editor's Note

This journal *Protein, Nucleic Acid and Enzyme* has become 18 years old since its birth. During that time, the research in biochemistry and related areas has grown rapidly covering a wide range of areas compared to other research field. People in the research area has been changed as well. Accordingly this journal has been more or less changed in its form and character. We learn about its change and a demand for this journal when we gather reader's cards which are inserted once a year in the magazine and when we directly visit a research office on a daily basis or when we cover an academic conference.

We are thrown criticism such as "In the recent magazines, taking the overall explanations as an example, each theme for overall explanations are too narrow. It is advisable, for example, to make it like "Biochemistry of Differentiation" or "Physiochemistry of Histone." Always give feedback," and, "I want to see sort of its thought process for each overall explanations." Furthermore, we were named in a certain medical journal and harshly commented on, "we cannot recommend that journal to our students unless they treat it directly connected to physiology." We think it is also good if we could consider their demands and criticisms were made in good will and absorb them to find a new direction. (U)

In October last year, the first volume (RNA version) was published and we were pressed to issue the DNA version as soon as possible from various quarters, however, finally we have finished its proofreading. We are not that confident if we could take advantage of promptness of a magazine.

When I looked at the proofed draft, I was first surprised at the fact that there were so many techniques for experiment. Even so, it is certain some important parts may be omitted in some cases. Nevertheless, the day when a book is published is the deadline to which various elements such as the convenience of a lot of writer, publishers, printing companies as well as the sales people are distilled, therefore, in a sense it is inevitable to face delay. Missing parts should be supplemented by this journal some time.

We requested the writers to include some specific and detailed cautions and tips. Of course we cannot guarantee that everything goes well if you follow it, however, we expect that this will be a useful ultimate reference book.

We would like to extend many thanks to Mr. Akira Ishihama, Mr. Reiji Okazaki, Mr. Yoshimasa Kyogoku and Mr. Susumu Nishimura who served as editorial committees of this book for their tremendous cooperations in each stage. Above all, we would like to extend special thanks to Mr. Ishihama for the trouble of reading all of the manuscripts.

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